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Isolation of a primate embryonic stem cell line

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ABSTRACT **Embryonic stem cells have the ability to remain undifferentiated and proliferate indefinitely *in vitro* while maintaining the potential to differentiate into derivatives of all three embryonic germ layers. Here we report the derivation of a cloned cell line (R278.5) from a rhesus monkey blastocyst that remains undifferentiated in continuous passage for >1 year, maintains a normal XY karyotype, and expresses the cell surface markers (alkaline phosphatase, stage-specific embryonic antigen 3, stage-specific embryonic antigen 4, TRA-1-60, and TRA-1-81) that are characteristic of human embryonal carcinoma cells. R278.5 cells remain undifferentiated when grown on mouse embryonic fibroblast feeder layers but differentiate or die in the absence of fibroblasts, despite the presence of recombinant human leukemia inhibitory factor. R278.5 cells allowed to differentiate *in vitro* secrete bioactive chorionic gonadotropin into the medium, express chorionic gonadotropin α- and β-subunit mRNAs, and express α-fetoprotein mRNA, indicating trophoblast and endoderm differentiation. When injected into severe combined immunodeficient mice, R278.5 cells consistently differentiate into derivatives of all three embryonic germ layers. These results define R278.5 cells as an embryonic stem cell line, to our knowledge, the first to be derived from any primate species.**

Embryonic stem (ES) cells, derived from preimplantation embryos (1, 2), and embryonic germ (EG) cells, derived from fetal germ cells (3, 4), are undifferentiated, immortal cells capable of differentiating into derivatives of all three embryonic germ layers. Well-characterized ES and EG cells have been derived only from rodents (1, 2, 5, 6). Pluripotent cell lines have been derived from preimplantation embryos of several non-rodent species (7–10), but the developmental potentials of these cell lines remain poorly characterized. Mouse ES cells remain undifferentiated through serial passages when cultured in the presence of leukemia inhibitory factor (LIF) and differentiate in the absence of LIF (11). Mouse ES cells injected into syngeneic mice form teratocarcinomas that exhibit disorganized differentiation, with representatives of all three embryonic germ layers. Mouse ES cells combined with normal preimplantation embryos as chimeras and returned to the uterus participate in normal development (12). Because mouse ES cells can contribute to functional germ cells in chimeras, specific genetic changes can be introduced into the mouse germ line through the use of ES cell chimeras (13).

The mechanisms controlling differentiation of specific lineages can be studied with mouse ES cells grown *in vitro*; however, significant differences between early human and mouse development suggest that human development will be more accurately represented by primate ES cells. For example, human and mouse embryos differ in the timing of embryonic genome expression (14), in the structure and function of the fetal membranes and placenta (15), and in formation of an embryonic disc instead of an egg cylinder. Human embryonal carcinoma (EC) cells, which are pluripotent, immortal stem cells from teratocarcinomas, provide an important *in vitro* model for understanding human differentiation (16). Some EC cell lines can be induced to differentiate in culture (17), which results in the loss of specific cell surface markers [stage-specific embryonic antigen 3 (SSEA-3), SSEA-4, TRA-1-60, and TRA-1-81] and the appearance of new markers (16). When pluripotent human EC cells are injected into immunocompromised mice, they form teratocarcinomas, some with derivatives of all three embryonic germ layers. However, there are limitations to the use of human EC cells in the study of development. (*i*) The range of differentiation obtained from human EC cell lines is more limited than that obtained from mouse ES cells and varies widely between cell lines (18). (*ii*) All pluripotent human EC cell lines derived to date are aneuploid (19), suggesting EC cells may not provide a completely accurate representation of normal differentiation. (*iii*) Ethical considerations severely restrict the study of human embryos, often making it impossible to verify that *in vitro* results have significance in the intact embryo. None of these limitations would be present with nonhuman primate ES cell lines.

Here we report the isolation of an ES cell line (R278.5) from a rhesus monkey blastocyst. This cloned cell line remains undifferentiated and continues to proliferate for >1 year in culture, maintains a normal XY karyotype, and maintains the potential to differentiate into trophoblast and to derivatives of embryonic endoderm, mesoderm, and ectoderm. The morphology, cell surface markers, and growth factor requirements of these cells differ significantly from mouse ES cells but closely resemble human EC cells.

MATERIALS AND METHODS

Cell Line Isolation. Six days after ovulation, an azonal blastocyst was recovered by a nonsurgical uterine flush technique from a 15-year-old rhesus monkey (20). The trophectoderm was removed by immunosurgery (21) using a rabbit anti-rhesus spleen cell antiserum followed by exposure to guinea pig complement. The intact inner cell mass (ICM) was separated from lysed trophectoderm cells and plated on mouse embryonic fibroblasts [previously exposed to 3000 rads (1 rad = 0.01 Gy) γ-radiation] in medium consisting of 80% Dulbecco's modified Eagle medium (4500 mg of glucose per liter, with L-glutamine, without sodium pyruvate; GIBCO) with 20% fetal bovine serum (HyClone), 0.1 mM 2-mercaptoethanol (Sigma), 1% nonessential amino acid stock (GIBCO) (22), and 1000 units of cloned human LIF per ml (GIBCO). After 16 days of culture, a central mass of cells was removed from

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Abbreviations: CG, chorionic gonadotropin; ES, embryonic stem; EC, embryonal carcinoma; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; ICM, inner cell mass; LIF, leukemia inhibitory factor; RT-PCR, reverse transcription polymerase chain reaction; SCID, severe combined immunodeficiency; SSEA, stage-specific embryonic antigen.

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epithelial outgrowths, exposed for 3 min to 0.05% trypsin-EDTA (GIBCO), gently dissociated by pipetting through a micropipette, and replated on mouse embryonic fibroblasts. After 3 weeks of growth, colonies with a morphology resembling human EC cells were selected and expanded. At five passages, individual cells were selected by micropipette and plated in individual wells of a 96-well plate (Falcon) with mouse embryonic fibroblast feeder layers. One clone with a normal karyotype (R278.5) was expanded for further analysis.

Cell Surface Markers. R278.5 cells grown on a layer of mouse embryonic fibroblasts were used to examine the expression of cell surface markers. Alkaline phosphatase was detected histochemically following fixation of cells with 100% ethanol using "Vector red" (Vector Laboratories) as a substrate, as described by the manufacturer. The SSEA-1, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 antigens were detected by immunocytochemistry with specific primary monoclonal antibodies (gifts of Peter Andrews, University of Sheffield, U.K.) (16, 23–25) and localized with a biotinylated secondary antibody and then an avidin/biotinylated horseradish peroxidase complex (Vectastain ABC system, Vector Laboratories).

***In Vitro* Differentiation.** R278.5 cells were plated at low density (≈5000 cells/cm^2 of surface area) in the absence of fibroblasts on gelatin-treated four-well tissue culture plates (Nunc) in the same medium as that used for initial cell line isolation, but with 0–10^4 units of added human LIF per ml (GIBCO). The resulting differentiated cells were photographed 8 days after plating.

A mouse Leydig cell bioassay (26) was used to measure luteinizing hormone/chorionic gonadotropin (CG) activity in medium conditioned for 2 days either by undifferentiated R278.5 cells (at 80% confluence on fibroblast feeder layers) or by spontaneously differentiated R278.5 cells (cultured for 2 weeks after achieving confluence on fibroblast feeders). The relative levels of the mRNAs for α-fetoprotein and the α- and β-subunits of CG relative to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were determined by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) (27) using RNA from the same undifferentiated and differentiated cells. The PCR primers for human G3PDH (Clontech) do not amplify mouse G3PDH mRNA. Primers for human α-fetoprotein mRNA flank the seventh intron (5′ primer, 5′-GCTGGATTGTCTGCAGGATGGGGAA; 3′ primer, 5′-TCCCCTGAAGAAAATTGGTTAAAAT) and amplify a cDNA of 216 bp. Primers for the β-subunit of human CG flank the second intron (5′ primer, 5′-*ggatc*CACCGTCAACACCACCATCTGTGC; 3′ primer, 5′-*ggatc*CACAGGTCAAAGGGTGGTCCTTGGG) (nucleotides added to the CGβ sequence to facilitate subcloning are shown in italics) and amplify a cDNA of 262 bp. The primers for the CGα subunit were based on sequences of the first and fourth exon of the rhesus gene (28) (5′ primer, 5′-*gggaattc*GCAGTTACTGAGAACTCACAAG; 3′ primer, 5′-*gggaattc*GAAGCATGTCAAAGTGGTATGG) and amplify a cDNA of 556 bp. The identity of all cDNAs was verified by sequencing (not shown).

For RT-PCR, 1–5 μl of total R278.5 RNA was reverse transcribed, and 1–20 μl of reverse transcription reaction was subjected to the PCR in the presence of 2.5 μCi of deoxycytidine 5′-[α-^{32}P]triphosphate (1 Ci = 37 GBq; DuPont). The number of amplification rounds that produced linear increases in target cDNAs and the relation between input RNA and amount of PCR product were empirically determined. Following agarose gel electrophoresis, DNA bands of interest were cut out and radioactivity was determined by liquid scintillation spectroscopy. The ratio of cpm in a specific PCR product relative to cpm of G3PDH PCR product was used to estimate the relative levels of mRNAs among differentiated and undifferentiated cells.

Tumor Formation in Severe Combined Immunodeficient (SCID) Mice. In the passage immediately prior to SCID mouse injection (7 months after initial derivation of R278), karyotypes of R278.5 were confirmed as euploid. Approximately 5 × 10^5 R278.5 cells were injected either into the rear leg muscles (seven mice) or into the testis (two mice) of 8- to 12-week-old male SCID mice. The resulting tumors were fixed in 4% paraformaldehyde and examined histologically after paraffin embedding at 8–15 weeks of development.

RESULTS

The morphology and cell surface markers of R278.5 cells (Fig. 1*A*) more closely resembled human EC cells than mouse ES cells. R278.5 cells had a high nucleus/cytoplasm ratio and prominent nucleoli, but rather than forming compact, piled-up colonies with indistinct cell borders similar to mouse ES cells, R278.5 cells formed flatter colonies with individual, distinct cells. R278.5 cells expressed alkaline phosphatase activity and the cell surface antigens SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 (Fig. 2), cell surface markers characteristic of human EC cell lines (16). Although cloned human LIF was present in the medium at cell line derivation and for initial passages, R278.5 cells grown on mouse embryonic fibroblasts without exogenous LIF remained undifferentiated and continued to proliferate. R278.5 cells plated on gelatin-treated tissue culture plates without fibroblasts differentiated to multiple cell types or failed to attach and died, regardless of the presence or absence of exogenously added human LIF (Fig. 1*B*).

The mRNA for α-fetoprotein, a marker for endoderm, increased substantially with *in vitro* differentiation (Fig. 3). α-Fetoprotein is expressed by extra-embryonic (yolk sac) and embryonic (fetal liver and intestines) endoderm. Epithelial cells resembling extraembryonic endoderm were present in cells differentiated *in vitro* from R278.5 cells (Fig. 1*B*).

Luteinizing hormone activity, an indication of CG secretion and trophoblast differentiation, was present in culture medium collected from differentiated cells [3.89 milli-international units (mIU)/ml] but not in medium collected from undiffer-

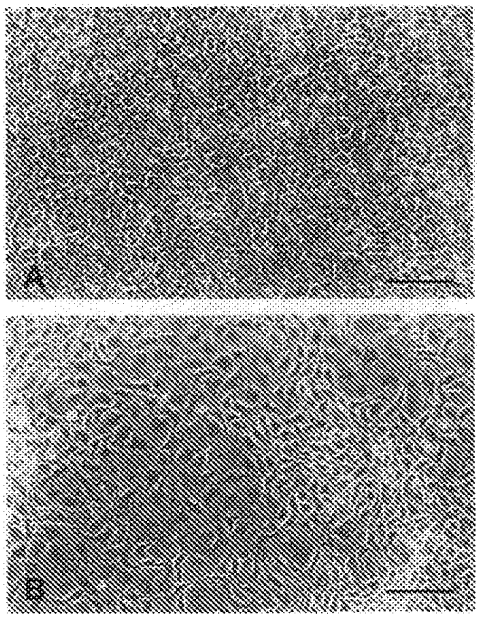

FIG. 1. Colony morphology and *in vitro* differentiation of cell line R278.5. (*A*) Undifferentiated R278.5 cells. Note the distinct cell borders, high nucleus to cytoplasm ratio, and prominent nucleoli. (Bar = 100 μm.) (*B*) Differentiated cells 8 days after plating R278.5 cells on gelatin-treated tissue culture plastic, with 10^3 units of added human LIF per ml. (Bar = 100 μm.)

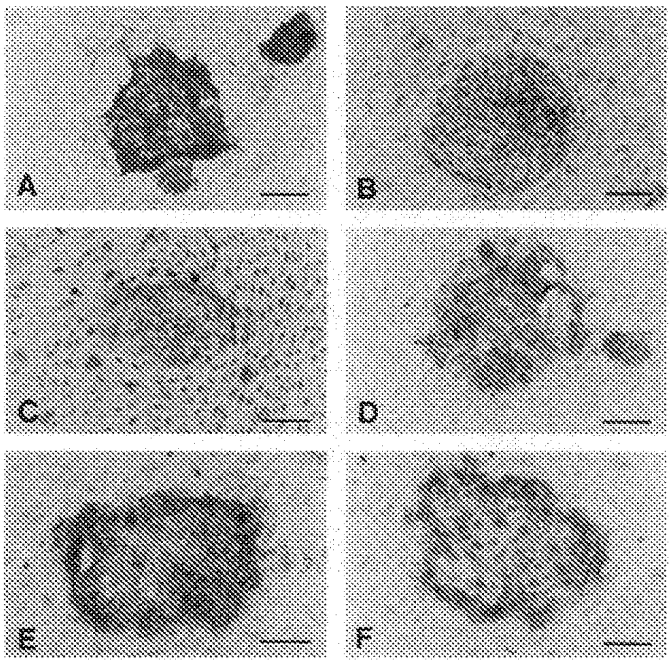

FIG. 2. Expression of cell surface markers by undifferentiated R278.5 cells. (*A*) Alkaline phosphatase. (*B*) SSEA-1. (*C*) SSEA-3. (*D*) SSEA-4. (*E*) TRA-1-60. (*F*) TRA-1-81. (Bars = 100 μm.) SSEA-3 staining of R278.5 cells was consistently weaker than the other positive antigens, and cell staining intensity varied within and between colonies.

entiated cells (<0.03 mIU/ml). The mRNAs for the CG subunits were readily detectable in the differentiated cells, although the relative level of the CGβ subunit mRNA was considerably lower than that for the CGα subunit (Fig. 4). The relative level of the CGα mRNA was quite low in undifferentiated cells, but the relative level was increased 23.9-fold after differentiation. The levels of the CGβ mRNA, on the other hand, increased only about 2-fold after differentiation for 2 weeks. Minor subpopulations of R278.5 cells differentiated even in the presence of fibroblasts, and the low level of α-fetoprotein, CGα, and CGβ mRNA present prior to the removal from fibroblasts could have been from these cells.

All SCID mice injected with R278.5 cells in either intramuscular or intratesticular sites formed tumors, and tumors in both sites demonstrated a similar range of differentiation. The

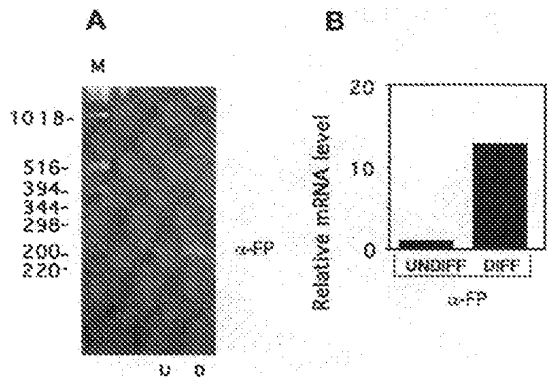

FIG. 3. Expression of α-fetoprotein mRNA. (*A*) PCR amplification of α-fetoprotein (αFP) cDNA from reverse-transcribed total RNA from undifferentiated (U) and differentiated (D) R278.5 cells. The DNA size markers (M) are indicated in bp. (*B*) The α-fetoprotein mRNA levels are expressed relative to the levels of the mRNA for G3PDH in each sample (not shown) as described in the text. Similar results were obtained in a second independent differentiation experiment.

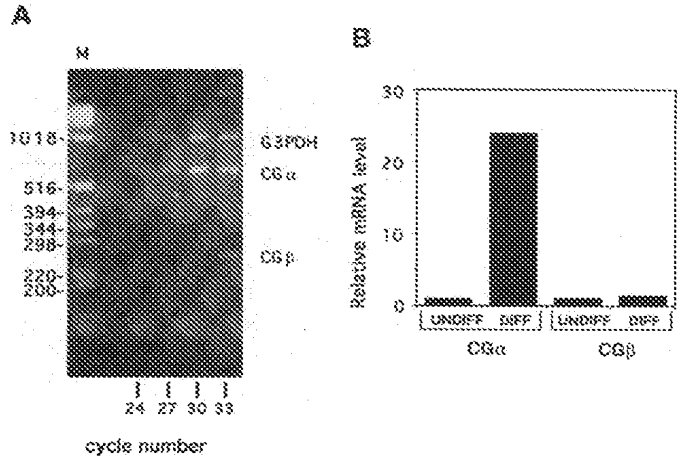

FIG. 4. Expression of CG subunit mRNA. (*A*) PCR amplification of cDNAs for G3PDH, CGα, and CGβ subunits from reverse-transcribed total RNA from differentiated R278.5 cells. The DNA size markers (M) are indicated in bp. (*B*) Relative levels of CGα and CGβ mRNAs in undifferentiated and differentiated R278.5 cells. Total RNA from cultured cells was analyzed for CG mRNA levels by RT-PCR and expressed relative to the levels of G3PDH mRNA. Similar results were obtained in a second independent differentiation experiment.

oldest tumors examined (15 weeks) had the most advanced differentiation, and all had abundant, unambiguous derivatives of all three embryonic germ layers, including ciliated columnar epithelium and nonciliated columnar epithelium (probable respiratory and gut epithelium; endoderm); bone, cartilage, smooth muscle, striated muscle (mesoderm); ganglia, other neural tissue, and stratified squamous epithelium (ectoderm), and other unidentified cell types (Fig. 5). Neural tissue included stratified cellular structures with remarkable resemblance to developing neural tube (Fig. 5*D*). Gut-like structures were often encircled by multiple layers of smooth muscle and were sometimes lined by villi with columnar epithelium interspersed with scattered mucus-secreting goblet cells (Fig. 5 *A* and *F*). Stratified squamous epithelium often contained well-differentiated hair follicles with hair shafts (Fig. 5*C*).

DISCUSSION

To our knowledge, there have been no previous reports of the isolation of a primate ES cell line. The characteristics that define R278.5 cells as ES cells include indefinite (>1 year) undifferentiated proliferation *in vitro*, maintenance of a normal karyotype, and potential to differentiate to derivatives of trophectoderm and all three embryonic germ layers. The development of complex structures in tumors in SCID mice with remarkable resemblance to normal hair follicles, neural tube, and gut demonstrates the ability of R278.5 cells to participate in complex developmental processes requiring coordinated interactions between multiple cell types. In the mouse embryo, the last cells capable of contributing to derivatives of trophectoderm and ICM are early ICM cells of the expanding blastocyst (29). The timing of commitment to ICM or trophectoderm has not been established for any primate species, but the potential of R278.5 cells to contribute to derivatives of both suggests that they most closely resemble early totipotent embryonic cells. The very limited ability of mouse ES cells to contribute to trophoblast in chimeras (30) suggests that the R278.5 cells represent an earlier developmental stage than mouse ES cells or that the ability of ICM cells to form trophectoderm persists longer in primates. Human EC cells share the ability of R278.5 cells to differentiate to trophoblast *in vitro* (16) and this potential may be a general distinguishing property of primate ES cell lines.

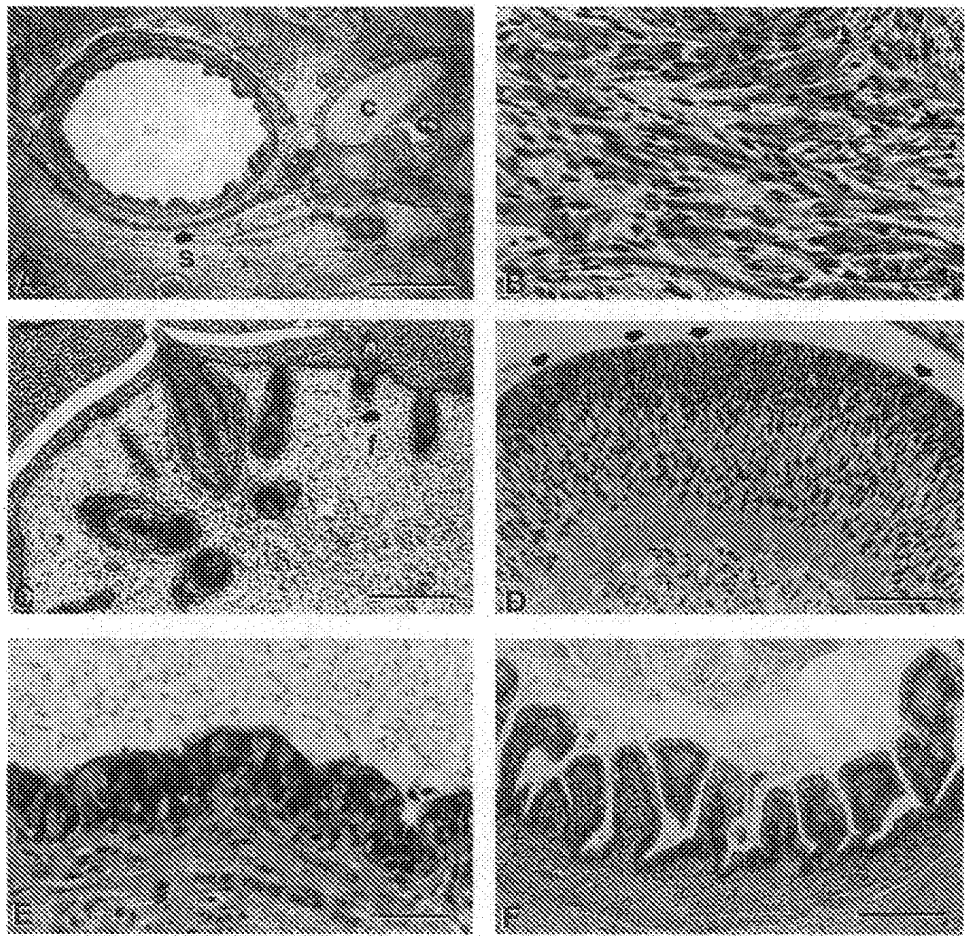

FIG. 5. Tumors formed by R278.5 cells injected into SCID mice and examined at 15 weeks. (*A*) Low-power field demonstrating disorganized differentiation of multiple cell types. A gut-like structure is encircled by smooth muscle (s), and elsewhere foci of cartilage (c) are present. (Bar = 400 μm.) (*B*) Striated muscle. (Bar = 40 μm.). (*C*) Stratified squamous epithelium with several hair follicles. The labeled hair follicle (f) has a visible hair shaft. (Bar = 200 μm.). (*D*) Stratified layers of neural cells in the pattern of a developing neural tube. An upper "ventricular" layer, containing numerous mitotic figures (arrows), overlies a lower "mantle" layer. (Bar = 100 μm.) (*E*) Ciliated columnar epithelium. (Bar = 40 μm.) (*F*) Villi covered with columnar epithelium with interspersed mucus-secreting goblet cells. (Bar = 200 μm.)

The only cells known to express the combination of markers alkaline phosphatase, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 other than R278.5 cells are human EC cells (16, 25, 31). This expression pattern contrasts with undifferentiated mouse ES and EC cells, which instead express SSEA-1 and do not express SSEA-3, SSEA-4, TRA-1-60, or TRA-1-81 (23, 24). Differentiation of human EC cells such as NTERA2 cl.D1 (17) results in the loss of SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 expression and an increased SSEA-1 expression (16). These antigens have yet to be studied in early human or nonhuman primate embryos, and their functions are unknown, but their shared expression by R278.5 cells and human EC cells suggests a close embryological similarity.

In the absence of fibroblast feeder layers, soluble LIF fails to prevent the differentiation of R278.5 cells or of feeder-dependent human EC cells (19). The factors that fibroblasts produce that prevent the differentiation of R278.5 cells or feeder-dependent human EC cells are unknown. Other factors that fail to support the growth of feeder-dependent human EC cells in the absence of feeder layers include oncostatin M and ciliary neurotrophic factor (19), both of which can substitute for LIF in preventing the differentiation of mouse ES cells (32, 33). A trypsin-sensitive factor from a human yolk sac carcinoma cell line (GCT 44) supports the growth of feeder-dependent human EC cells in the absence of fibroblasts, but the factor has not yet been purified (19).

Although exogenous LIF was added during the initial derivation of R278.5 cells, the cell line is now routinely passaged without added LIF. We have also recently derived two additional cell lines (R366 and R367) from four additional rhesus blastocysts, using the same techniques as described for R278.5 cells, but without added LIF (data not shown). R366 and R367 cells have normal karyotypes and continue to proliferate *in vitro* for at least 3 months. R366 and R367 cell lines have not yet been tested for tumor formation in SCID mice, but they are indistinguishable from R278.5 cells in undifferentiated morphology, growth characteristics, and *in vitro* differentiation in the absence of feeder layers.

The differentiation of R278.5 cells to trophoblast was demonstrated by the expression of CGα and CGβ subunit mRNAs and the secretion of bioactive CG into the culture medium by differentiated ES cells. We were surprised to note that while the relative levels of the CGα subunit were increased >20 times in differentiated cells, the relative levels of the CGβ subunit only changed about 2-fold. The fact that CG secretion increased substantially with differentiation may mean that under our *in vitro* culture conditions, expression of the CGα subunit is limiting for CG secretion. CGβ subunit mRNA is detectable in human preimplantation embryos as early as the eight-cell stage, which is before trophectoderm differentiation (34), consistent with a low level of CGβ mRNA expression in undifferentiated R278.5 cells. Although there may be some coordinate mechanisms regulating CGα and CGβ gene transcription in the placenta (35), it is clear that there is differential regulation of these genes *in vitro* and *in vivo* (36). Since the expression of the CGβ subunit is also divergent among villous

and extravillous trophoblasts (37), further studies are needed to determine the phenotype of the trophoblasts derived from R278.5 cells.

Primate ES cells will be particularly useful for *in vitro* developmental studies of lineages that differ substantially between humans and mice. However, the most accurate *in vitro* model of the differentiation of human tissues would be provided by human ES cells. In one published report, ICM-derived cells from spare *in vitro* fertilized human embryos were cultured with LIF in the absence of feeder layers, and, although alkaline phosphatase positive cells proliferated, they failed to survive beyond two passages (38). These results suggest that soluble LIF alone will not prevent the differentiation of human ES cells, just as it fails to prevent the differentiation of rhesus ES cells. The growth of rhesus monkey ES cells in culture conditions that support feeder-dependent human EC cells suggests that similar conditions may support human ES cells.

Human ES cells would offer exciting new possibilities for transplantation medicine. Because ES cells have the developmental potential to give rise to all adult cell types, any disease resulting from the failure of specific cell types would be potentially treatable through the transplantation of differentiated cells derived from ES cells. Because ES cells are immortal cell lines, they could be genetically manipulated prior to differentiation either to reduce immunogenicity or to give them new properties to combat specific diseases. Rhesus monkey ES cells and rhesus monkeys will be invaluable for testing the safety and efficacy of the transplantation of specific cell types for the treatment of specific diseases. Because of the range of diseases potentially treatable by this approach, elucidating the basic mechanisms controlling the differentiation of primate ES cells has dramatic clinical significance.

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Immunology

Hemato-lymphoid *in vivo* reconstitution potential of subpopulations derived from *in vitro* differentiated embryonic stem cells

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ABSTRACT **During differentiation *in vitro*, embryonic stem (ES) cells generate progenitors for most hemato-lymphoid lineages. We studied the developmental potential of two ES cell subpopulations that share the fetal stem cell antigen AA4.1 but differ in expression of the lymphoid marker B220 (CD45R). Upon transfer into lymphoid deficient mice, the $B220^+$ population generated a single transient wave of IgM^+ IgD^+ B cells but failed to generate T cells. In contrast, transfer of the $B220^-$ fraction achieved long-term repopulation of both T and B lymphoid compartments and restored humoral and cell-mediated immune reactions in the recipients. To assess the hemato-lymphopoietic potential of ES cell subsets in comparison to their physiological counterparts, cotransplantation experiments with phenotypically homologous subsets of fetal liver cells were performed, revealing a more potent developmental capacity of the latter. The results suggest that multipotential and lineage-committed lymphoid precursors are generated during *in vitro* differentiation of ES cells and that both subsets can undergo complete final maturation *in vivo*.**

Murine embryonic stem (ES) cells can spontaneously differentiate *in vitro* to structures resembling yolk sac blood islets (1) and, therefore, have become a model system for the study of cellular and molecular aspects of hematopoiesis (for review, see ref. 2). Initial reports on differentiating ES cells described the presence of hematopoietic cytokines and their respective receptors (3) and of myeloid (4, 5) and erythroid (1) progenitors. In the latter, the sequential expression of hemoglobin genes resembled the transition of "primitive" to "definitive" erythroid cells *in vivo* (6–8). Furthermore, the observation of angiogenesis in embryoid bodies (9) further underlines the similarities between ES differentiation *in vitro* and yolk sac development *in vivo* (extensively reviewed in ref. 10). More recently, relatively mature lymphoid progenitors have been demonstrated, either within *in vitro*-derived embryoid bodies (11) or by cocultivation on stroma cell lines (12). In addition to these *in vitro* approaches, unseparated differentiated ES cells have been shown to restore the lymphoid compartment of SCID (13, 14) or Rag-deficient mice (15). The capacity of ES cells to develop *in vitro* into virtually all hematopoietic lineages suggests the presence of a hematopoietic stem cell (HSC) or a primitive multilineage progenitor in these culture systems. Indeed, during a limited time span of ES cell differentiation, primitive multilineage progenitors have recently been isolated (16). In addition, by cocultivating ES cells with stroma cell lines in medium supplemented with various cytokines, a stem cell-like activity has been described (17).

In our previous studies of ES cell-derived lymphopoiesis, uncommitted hematopoietic precursor cells appeared to coexist with relatively advanced progenitors for both T and B cell lineages. To further characterize these stages in hemato-lymphopoiesis, it was our aim to identify ES cell subpopulations that harbor the different types of precursor activities. Moreover, we asked the question which of these progenitor/precursor populations gave rise to the lymphoid reconstitution reported by other groups upon *in vivo* transfer of unseparated ES cells (13–15). To this end, phenotypically defined subpopulations of *in vitro*-differentiated ES cells were tested for their ability to reconstitute the lymphoid system of Rag-deficient mice. The results identify two distinct subsets of *in vitro*-differentiated ES cells, harboring lymphoid potential with distinct developmental capacities. The direct comparison of these subsets to progenitors developing *in vivo* will allow the further definition of the developmental capacity of ES cell-derived hematopoiesis.

MATERIALS AND METHODS

Animals and Cell Lines. Rag-1-deficient mice, backcrossed to C57/BL6 ($Ly\text{-}5.2^+$, $Ly\text{-}9.1^-$), were maintained under specific-pathogen-free conditions in the animal facility of our institute. Breeding stocks of C57/BL6 Ly-5.1 ($Ly\text{-}5.1^+$, $Ly\text{-}9.1^-$) were provided by H.-R. Rodewald (Basel Institute for Immunology, Basel, Switzerland). Fetal liver (FL) was isolated from timed matings of C57/BL6 Ly-5.1 mice. The day of the vaginal plug was counted as day 0.5 of pregnancy. The C57/BL6-derived ES cell lines Bl6-III or 129/Sv-derived D3/M were maintained in an undifferentiated state by culture on a monolayer of mitomycin C-inactivated embryonic fibroblasts.

Differentiation of ES Cells and Preparation of Cells. For differentiation cultures, ES cells were dissociated by trypsinization and cultured in Iscove's modified Dulbecco's medium (Biochrom, Berlin) supplemented with 15% fetal calf serum and 4.5×10^{-4} M monothioglycerol. For the induction of differentiation, ES cells were plated in gas-permeable 60-mm hydrophobic culture dishes (Heraeus) at a concentration of $0.8–1.4 \times 10^4$ cells per ml in a total volume of 5 ml. In some experiments embryoid bodies were induced by culturing 15–20 ES cells in 20 μl in inverted hanging drops. Embryoid bodies were transferred after 2–3 days and seeded on gas-permeable culture dishes. During the whole differentiation period, cultures were maintained at 37°C in an atmosphere of 7.5% CO_2/5% O_2 by using an incubator with adjustable oxygen content (Heraeus). After 15 days of differentiation, embryoid bodies were harvested and dissociated by gentle digestion with collagenase (0.1 unit/mg) and dispase (0.8 unit/mg; Boehringer Mannheim) in PBS. Cells were labeled with purified anti-CD45 monoclonal antibody (mAb) M1/9.3.4.HL.2, followed by goat anti-rat immunoglobin (Ig) conjugated to paramagnetic microbeads (Miltenyi, Bergisch Gladbach, Germany), and enriched by single passage over a MACS (Miltenyi) system. Enrichment was found to increase the number of $CD45^+$ cells from 21% (range: 16.7–32.5%) to 48% (range: 41.2–57.3%), as

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Abbreviations: ES cell, embryonic stem cell; FL, fetal liver; HSC, hematopoietic stem cell; AGM region, aorta–gonad–mesonephros region.

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analyzed by flow cytometry. FL cells were prepared similarly, except that the initial digestion step was omitted. Bone marrow, spleen, thymus, and lymph node cells of reconstituted animals were prepared by standard procedures. Bone marrow and lymph node cells were depleted for surface IgM^+ cells by incubation with a biotinylated goat anti-mouse IgG/IgM serum (Jackson-Dianova, Hamburg, Germany) detected by streptavidin-paramagnetic microbeads (Miltenyi), followed by separation on MACS. Both preparations were found to contain less than 1% IgM^+ cells.

Staining, Flow Cytometry, and Cell Sorting. For further purification of MACS-enriched ES or FL populations, cells were purified by flow cytometry. After blocking nonspecific antibody binding with normal rat Ig, cells were stained with fluorescein isothiocyanate-labeled anti-B220 mAb (RA3–6B2, PharMingen) and biotinylated mAb AA4.1 (18), followed by streptavidin-phycoerythrin (Southern Biotechnology Associates) in PBS/5% fetal calf serum on ice. Cells were separated into $AA4.1^+$ $B220^-$ and $AA4.1^+$ $B220^+$ fractions by using a FACStar Plus cell sorter (Becton Dickinson). Sorted cells were found to be ≥98% pure upon reanalysis. For phenotypic analysis, single cell suspensions were stained with mAbs as indicated. Dead cells were excluded from analysis on a FACScan (Becton Dickinson) by propidium iodide (1 μg/ml) counterstaining. For each diagram, at least 2×10^4 cells were analyzed on a logarithmic scale.

Cell Transfer into Rag-1-Deficient Animals. Sorter-purified cell populations were injected intravenously in the lateral tail vein of 4- to 6-week-old Rag-$1^{-/-}$ mice in 100 μl of PBS. To facilitate hematopoietic development *in vivo*, animals were sublethally irradiated with 400 rads (1 rad = 0.01 Gy) from an x-ray source prior to transfer. Mice were housed in a positive-pressure cabinet and received neomycin (0.1 g/100 ml) and Borgal (10% final concentration; Hoechst) for the first 2 weeks after irradiation. Injected cell numbers are indicated in the text.

ELISAs. Ig production in the serum of reconstituted mice was tested by standard ELISA. Briefly, Nunc-Immuno plates (Nunc) were coated with goat anti-mouse Ig $F(ab)_2$ (Jackson-Dianova) or purified mAbs from allotype-specific hybridomas RS3.1 (anti-mouse IgM^a) or AF6 (anti-mouse IgM^b). Serum samples were serially diluted and detected with alkaline phosphatase-conjugated goat anti-mouse IgM or IgG (Southern Biotechnology Associates). Results were quantified by using external standards (all from Southern Biotechnology Associates). Relative serum titers of different antibody isotypes (IgM, IgG1, IgG2a, IgG2b, and IgG3) were determined by incubating serum samples on trinitrophenyl-ovalbumin (50 μg/ml)-coated plates, followed by detection with alkaline-phosphatase-conjugated isotype-specific secondary antibodies (all from Southern Biotechnology Associates). Absorption was read at 405/490 nm by using *p*-nitrophenyl phosphate (Boehringer Mannheim) as a substrate.

Immunization and Proliferation Experiments. Reconstituted Rag-$1^{-/-}$ mice were injected s.c. (base of the tail) with 100 μl of an emulsion of 100 μg of trinitrophenyl-ovalbumin in PBS and incomplete Freund's adjuvant. Two weeks later, immunization was repeated s.c. as well as i.p. Serum was taken at the indicated times before and after booster immunization. Mesenteric and inguinal lymph nodes were prepared and depleted of IgM^+ cells as described above. For proliferation assays, 10^5 T cells were stimulated with Con A (5 μg/ml) and ovalbumin (100 μg/ml or 250 μg/ml) for 48 h and pulsed for 8 h with 0.5 μCi of [^{3}H]thymidine (1 Ci = 37 GBq). All results represent the means of triplicates, with standard deviations shown by error bars.

RESULTS

Restoration of the Lymphoid Compartment of Rag-1-Deficient Mice by Transfer of *in Vitro*-Differentiated ES Cell Subsets. Upon *in vitro* differentiation, ES cells express genes and surface antigens indicative for hemato-lymphoid commitment (4, 11). We first addressed the question whether a combination of surface molecules could be used to enrich, and/or to distinguish between, different stages of hemato-lymphopoietic development. To concentrate on hematopoietic cells, day 15 differentiated ES cells were first enriched for CD45 (T200) by MACS. The enriched population was then analyzed by flow cytometry for expression of B220 and AA4.1. We have reported the expression of B220 (CD45R) on a small proportion (≈ 3%) of ES cells differentiated *in vitro* for 15–20 days (11). The AA4.1 antibody has been described to recognize fetal HSCs (19) and primitive multilineage progenitors (20). Double staining for these markers reveals four distinct populations (21): The majority is negative for both markers (≈57%), a small population (≈3%) expresses B220 only, and the remaining $AA4.1^+$ cells can be divided into a $B220^-$ (≈32%) fraction and a $B220^+$ (≈8%) fraction. In preliminary transfer experiments, the B220 single-positive population did not give rise to any lymphoid cell type *in vivo* and was, therefore, not further investigated. The $AA4.1^+$ $B220^-$ and $AA4.1^+$ $B220^+$

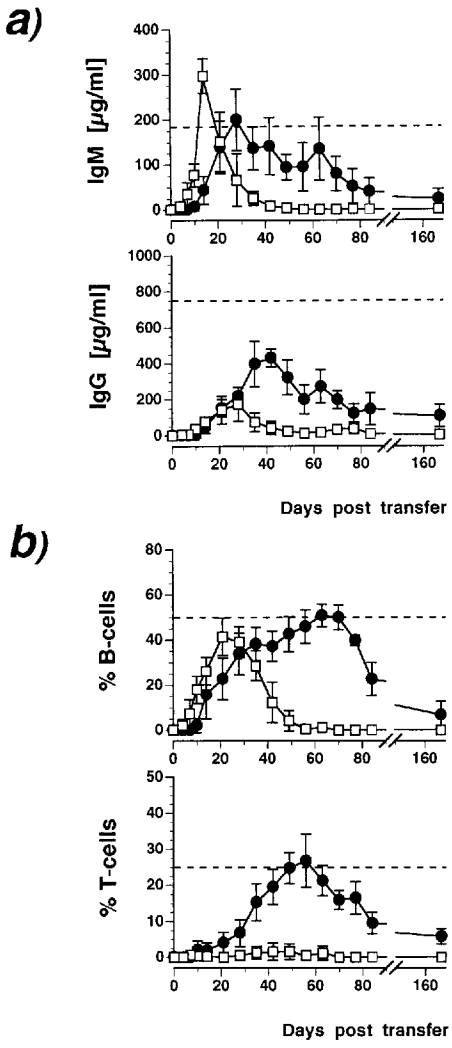

FIG. 1. Kinetics of peripheral lymphoid reconstitution in Rag-1-deficient mice with ES-derived lymphoid progenitors. At indicated times after transfer, blood was analyzed for serum IgM and IgG (*a*) and for peripheral blood B and T cells (*b*). (*a*) Appearance of IgM (*Upper*) and IgG (*Lower*) after the injection of 10^4 $AA4.1^+$ $B220^+$ (□) or 10^4 $AA4.1^+$ $B220^-$ (●) ES-derived progenitors. Cellular reconstitution was assessed by two-color flow cytometry for IgM/CD45 (*b Upper*) or CD3/CD45 (*b Lower*) by using the same samples as in *a*. Results are given either as total amount of Ig (*a*) or as numbers in percent relative to total ($CD45^+$) leukocytes in blood. Dashed lines indicate wild-type levels of Ig and B or T cells in C57/BL6 mice for comparison. The data represent results (mean ± SD) from three experiments with four to six animals per group.

Table 1. Development of AA4.1$^+$ B220$^+$ and AA4.1$^+$ B220$^-$ *in vitro*-differentiated ES cells into mature lymphocytes upon transfer into Rag-1$^{-/-}$ mice

		Flow cytometry analysis, % positive cells					
		8 weeks after transfer		12 weeks after transfer		24 weeks after transfer	
		AA4.1$^+$ B220$^+$ (experiments 1, 2, and 4; n = 8)	AA4.1$^+$ B220$^-$	AA4.1$^+$ B220$^+$ (experiments 1, 2, 3, and 5; n = 11)	AA4.1$^+$ B220$^-$	AA4.1$^+$ B220$^+$ (experiments 3 and 5; n = 5)	AA4.1$^+$ B220$^-$
Thymus	TCR $\alpha\beta$	0.0	38.2	0.0	33.8	0.0	24.2
	TCR $\gamma\delta$	0.0	6.9	0.0	1.8	0.0	2.5
Spleen	CD3	0.4	17.8	0.2	22.4	0.1	12.6
	IgM	19.4	20.4	9.6	22.6	0.8	12.2
	IgD	16.8	18.3	6.8	20.9	0.6	13.6
Bone marrow	CD3	0.1	0.2	0.0	0.1	0.2	0.0
	IgM	7.8	8.9	1.6	9.4	0.9	3.8
	IgD	5.9	0.8	0.9	1.2	0.4	0.7

Sublethally irradiated Rag-1$^{-/-}$ mice were reconstituted by intravenous injection of 10^4 cells of the indicated subsets of day 15 ES cells. Data from five experiments with ES cell line Bl6-III (C57/BL6 derived, experiments 1 and 2) or D3/M (129/Sv, experiments 3, 4, and 5) are shown. In every experiment, each group contained initially five or six animals. Results are given as the mean of antigen expression as determined by flow cytometry. The origin of the ES cells and the number of animals analyzed are given for every time separately. TCR, T cell receptor.

fractions were isolated by preparative flow cytometry and transferred into Rag-1-deficient mice. These mice are convenient hosts because, in contrast to lethally irradiated mice, their long-term survival does not require HSC transfer. In addition, because of the complete block of lymphocyte maturation, all mature lymphocytes in these mice are donor-derived. To facilitate hematopoietic reconstitution, Rag-1$^{-/-}$ recipients were sublethally irradiated with 400 rad and subsequently i.v.-injected with 10^4 AA4.1$^+$ B220$^-$ or AA4.1$^+$ B220$^+$ ES cells.

Transfer of the AA4.1$^+$ B220$^+$ population rapidly reconstituted serum IgM, reaching C57BL/6 wild-type levels within 2 weeks (Fig. 1*a*). These IgM levels quickly returned to background and became undetectable at 8 weeks. IgG was restored by the AA4.1$^+$ B220$^+$ population only to a small extent and with delayed

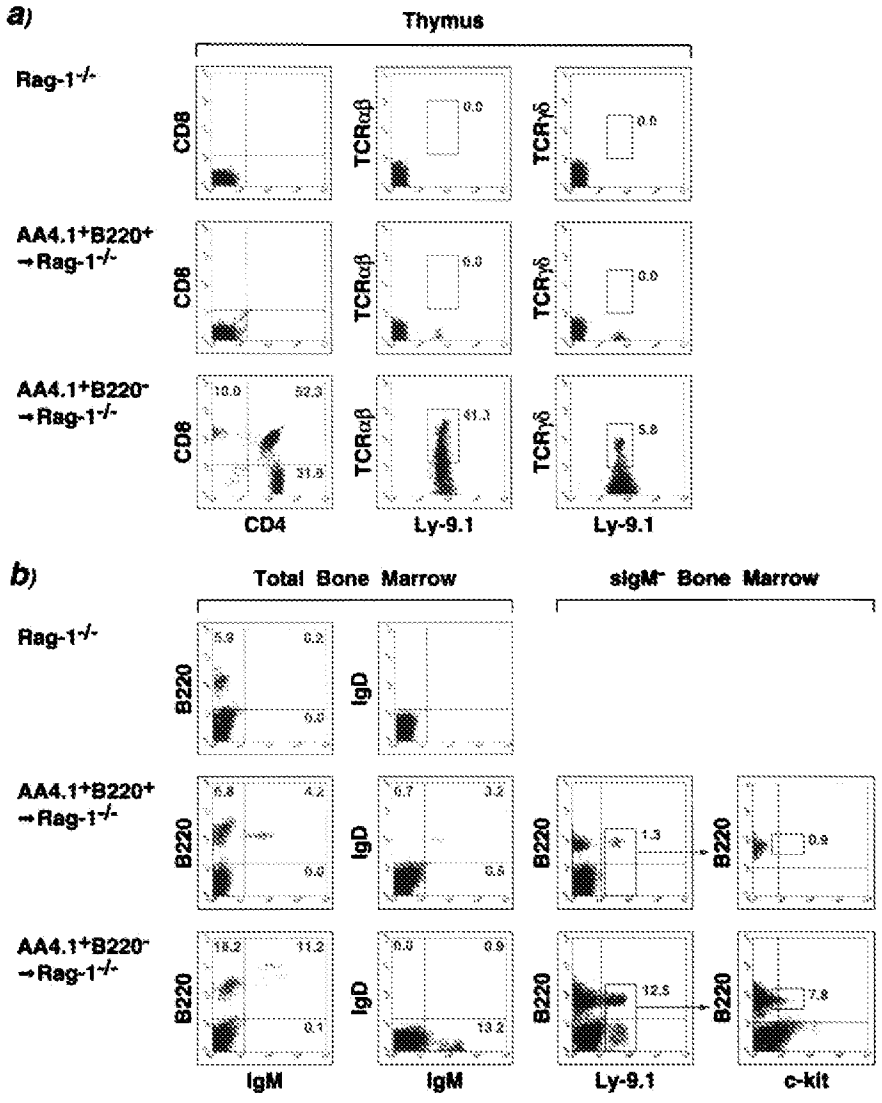

FIG. 2. Lymphoid reconstitution of Rag-1$^{-/-}$ mice by subsets of *in vitro*-differentiated ES cells. ES cells were separated in AA4.$^+$ B220$^+$ and AA4.1$^+$ B220$^-$ populations as detailed. Eight weeks after intravenous injection of 10^4 cells of the respective populations, mice were analyzed for lymphoid reconstitution in the thymus (*a*) or in the bone marrow (*b*). Thymocytes were stained with CD4/CD8 or T cell receptor (TCR) $\alpha\beta$, TCR $\gamma\delta$, and the donor-specific marker Ly-9.1. (*b*) Total bone marrow was investigated for the presence of IgM- or IgD-expressing cells. To detect donor-derived pro-B cells, bone marrow was depleted for surface IgM$^+$ cells and stained simultaneously for B220, c-kit, and Ly-9.1. Numbers given in the individual quadrants and gates indicate percentages.

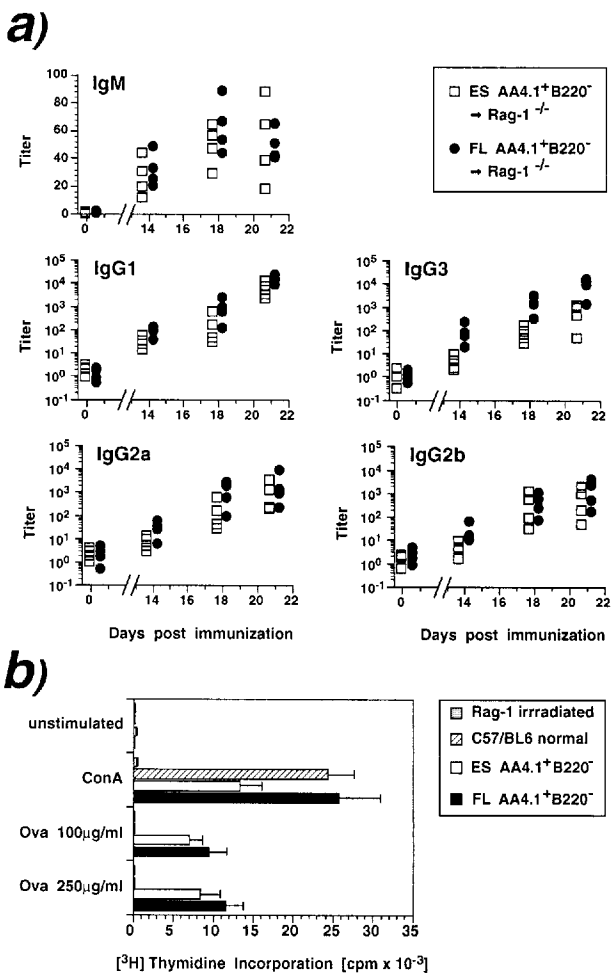

FIG. 3. ES-derived lymphoid progenitors acquire full-functional competence after *in vivo* transfer. AA4.1$^+$ B220$^-$ cells (5×10^4) from ES cells differentiated for 15 days or isolated from FL were transferred into Rag-1$^{-/-}$. Recipients were immunized with trinitrophenyl-ovalbumin by a priming injection 8 weeks after transfer and a booster injection 2 weeks later. (*a*) Antigen-specific titer for each individual mouse (n = 4, two experiments) reconstituted with ES (□) or FL-derived (●) cells. (*b*) Three weeks after immunization, surface-IgM-depleted lymph node cells were stimulated with Con A (5 μg/ml) or indicated concentrations of ovalbumin and tested for proliferation. Results are the mean ± SD of triplicate samples.

kinetics compared with IgM (Fig. 1*a*). Thus, AA4.1$^+$ B220$^+$ cells gave rise to a single and transient wave of serum Ig. In contrast, transfer of the AA4.1$^+$ B220$^-$ fraction reconstituted serum Ig with slower kinetics, reaching maximal levels within 4 weeks (IgM) or 5 weeks (IgG) (Fig. 1*a*). Serum levels declined more slowly and both Ig classes were still detectable 24 weeks after transfer, i.e., for the entire period of observation. The analysis of peripheral blood lymphocytes in Rag-1$^{-/-}$ recipients reconstituted with the AA4.1$^+$ B220$^+$ fraction revealed the complete absence of T cells and the emergence of a single wave of B cells (Fig. 1*b*). In contrast, the AA4.1$^+$ B220$^-$ subset developed into both T and B lymphoid lineages, which remained detectable until week 24 (Fig. 1*b*). Progeny of either ES cell subset were able to repopulate secondary lymphoid organs, including spleen (Table 1) and lymph node (data not shown). As in peripheral blood, AA4.1$^+$ B220$^+$ derived cells were detectable in the spleen for up to week 12 but not thereafter. In bone marrow, they gave rise to IgM$^+$ IgD$^+$ cells only (Fig. 2*b*), suggesting reentry from the circulation rather than local development. AA4.1$^+$ B220$^-$-derived cells were detectable in the spleen until week 24 (Table 1), and most were IgM single-positive in the bone marrow (Fig. 2*b*), suggesting local development. These experiments were performed with the congenic C57/BL6-derived ES cell line Bl6-III and allogenic 129/Sv-derived D3/M cells, without apparent strain-dependent variations in lymphoid reconstitution. Thus, these results are consistent with the notion that the AA4.1$^+$ B220$^+$ subset contains a relatively mature precursor committed to the B cell lineage that is unable to self renew, whereas the AA4.1$^+$ B220$^-$ population harbors multilineage progenitor activity giving rise to long-term reconstitution of the entire lymphoid compartment.

To examine the contribution of ES-derived cells to earlier stages of lymphoid development, it was necessary to use genetic markers that distinguish donor (129/Sv, marker Ly-9.1)-derived cells from host (Rag-1$^{-/-}$ mice backcrossed to C57/BL6, Ly-9.1$^-$). Bone marrow was examined after depletion of surface IgM$^+$ cells (Fig. 2*b*). Donor-derived B220$^+$ c-kit$^+$ pro-B cells were completely absent in animals reconstituted with AA4.1$^+$ B220$^+$ ES cells. In contrast, the AA4.1$^+$ B220$^-$ fraction contributed to the most immature B220$^+$ c-kit$^+$ pro-B subset and the B220$^-$ c-kit^{high} fraction, known to contain multilineage progenitors (Fig. 2*b*). In some animals (5 of 21 mice analyzed after 8 weeks), we detected various amounts of donor-derived bone marrow myeloid cells (data not shown). Consistent with the appearance of peripheral blood T cells, AA4.1$^+$ B220$^-$ ES cells developed into αβ as well as γδ T cells in the thymus of reconstituted mice (Fig. 2*a*). No intrathymic T cell development was observed with the AA4.1$^+$ B220$^+$ fraction (Fig. 2*a* and Table 1). Interestingly, intrathymic T cell development was initially biased toward the γδ lineage, although the αβ lineage predominated at all times (Table 1). Collectively, the data in Figs. 1 and 2 and Table 1 demonstrate that both AA4.1$^+$ B220$^+$ and AA4.1$^+$ B220$^-$ ES-derived populations contain precursor activity for B cells, whereas only AA4.1$^+$ B220$^-$ ES-derived cells give rise to T and B cells. The data suggest that the two subsets of differentiated ES cells represent distinct stages of lymphoid differentiation, the AA4.1$^+$ B220$^+$ subset being more advanced than the AA4.1$^+$ B220$^-$ cells. The long-term T cell development in the thymus, in combination with the contribution to the B220$^-$ c-kit^{high} population in the bone marrow, argues for the presence of an early multilineage progenitor within the AA4.1$^+$ B220$^-$ ES cells.

ES-Derived Lymphocytes Acquire Full Functional Competence *in Vivo*. We reconstituted Rag-1$^{-/-}$ hosts with either 5×10^4 ES-derived AA4.1$^+$ B220$^-$ cells or the same number of embryonic day 14.5 FL cells, which were isolated *ex vivo* by using an identical protocol. Reconstitution was verified by serum Ig levels and recipient mice were immunized with trinitrophenyl-ovalbumin by a priming injection 8 weeks after transfer and a booster injection 2 weeks later. Both ES- and FL-derived populations mounted similar humoral immune responses (Fig. 3*a*) in IgM and all IgG classes except for IgG3, which remained lower in the case of ES cells (mean titer 21 days after immunization: 607) compared with FL (mean: 9,400). Moreover, the proliferative responses of IgM-depleted lymph node T cells to ovalbumin were of similar magnitude in both groups of reconstituted mice (Fig. 3*b*), demonstrating the presence of antigen-reactive T cells. These results indicate that ES-derived progenitors can undergo final lymphoid differentiation *in vivo*, thereby acquiring full functional capacity.

Competitive Lymphoid Reconstitution of Rag-Deficient Mice with ES- and FL-Derived Populations. Next we tested the repopulation capacity of ES-derived AA4.1$^+$ B220$^-$ cells in competition with the homologous population isolated from embryonic day 14.5 FL. AA4.1$^+$ B220$^-$ cells were isolated from C57/BL6 FL (Ly-5.1$^+$, Ly-9.1$^-$, IgM^b) and from D3/M ES cells derived from 129/Sv mice (Ly-5.2$^+$, Ly-9.1$^+$, IgM^a) and were transferred into Rag-1$^{-/-}$ mice backcrossed to C57/BL6 (Ly-5.2$^+$, Ly-9.1$^-$) separately or in mixtures at various ratios (Table 2). The allotype titers in the serum of reconstituted animals showed a considerable bias toward the FL-derived Ig allotype for all three cell mixtures transferred (Table 2). A similar predominance of the

Table 2. Competitive lymphoid repopulation of Rag-$1^{-/-}$ recipients with ES- or FL-derived AA4.1^+ B220$^-$ subpopulations

			Group 1	Group 2	Group 3	Group 4	Group 5	Origin
Allotype titers (% of respective allotype)								
Serum		IgM[a]	100.0	31.9	16.4	1.2	0.1	ES
		IgM[b]	0.1	65.0	83.9	88.2	100.0	FL
Flow cytometry analysis (% positive cells)								
Thymus	CD4$^+$ CD8$^+$	Ly-9.1$^+$	90.1	11.3	8.1	5.4	1.4	ES
		Ly-5.1$^+$	3.1	85.6	90.7	92.3	94.1	FL
	CD4$^-$ CD8$^-$	Ly-9.1$^+$	15.1	8.5	5.8	2.4	1.3	ES
		Ly-5.1$^+$	1.6	8.4	19.1	21.6	25.2	FL
		Ly-5.2$^+$	83.6	83.9	76.1	76.6	73.9	Rag-$1^{-/-}$
Bone marrow	B220$^+$ CD25$^+$	Ly-9.1$^+$	86.8	7.5	6.9	1.6	0.9	ES
		Ly-5.1$^+$	1.7	84.8	86.4	92.3	93.5	FL
	B220$^+$ c-kit$^+$	Ly-9.1$^+$	6.7	5.1	0.8	1.1	0.5	ES
		Ly-5.1$^+$	1.4	9.1	16.4	19.5	17.6	FL
		Ly-5.2$^+$	92.0	86.4	82.9	79.6	82.0	Rag-$1^{-/-}$

Mixtures of AA4.1^+ B220$^-$ cells were transferred into Rag-$1^{-/-}$ mice at various ratios. Ratios of ES AA4.1^+ B220$^-$ cells to FL AA4.1^+ B220$^-$ cells are as follows: Group 1, 50,000/0; 2, 45,000/5,000; 3, 25,000/25,000; 4, 5,000/45,000; 5, 0/50,000. Two experiments were performed with two animals in each experimental group. Eight weeks after transfer, animals were sacrificed and analyzed. Allotype titers were determined by using an allotype-specific ELISA. Results are given in percent, setting serum levels in animals receiving exclusively ES or FL populations at 100%. Lymphoid populations in the thymus and the bone marrow were assessed by three-color flow cytometry, staining for the indicated subpopulations with either Ly-9.1 (indicative for ES origin) or Ly-5.1 (FL).

progeny of FL is observed among CD4$^+$ CD8$^+$ thymocytes and among B220$^+$ CD25$^+$ bone marrow cells (Table 2). Both populations are absent in Rag-deficient mice (22–24) and originate exclusively from the inoculum (Fig. 4). When the ratio of ES- to FL-derived cells in the inoculum was 9:1, the ratios of the progenies in either thymus (Fig. 4*a*) or bone marrow (Fig. 4*b*) was shown to be the inverse (1 ES-derived to 8.5 FL-derived progeny). The predominance of FL is less striking in lymphoid populations prior to the genetic block in Rag-$1^{-/-}$ recipients. CD4$^-$ CD8$^-$ thymocytes and B220$^+$ c-kit$^+$ bone marrow cells of ES or FL origin occurred in comparable proportions in animals that received a 9:1 mixture of ES/FL cells (Fig. 4). In all experiments, the predominance of FL was more pronounced in bone marrow than in thymus (Table 2). Thus, these results clearly demonstrate a superior reconstitution potency for FL compared with ES cells but nevertheless define ES-derived progenitors as capable of developing into mature lymphocytes even in a competitive protocol.

DISCUSSION

We have examined the *in vivo* reconstitution potential of phenotypically defined subsets of differentiated ES cells by transfer into sublethally irradiated Rag-1-deficient mice. The two transplanted populations were found to represent two distinct stages of lymphoid development on the basis of (*i*) the lymphoid lineages restored, (*ii*) the kinetics of reconstitution, and (*iii*) the persistence, tissue distribution, and subset relationships of the progeny of ES-derived cells.

The population defined by the combined expression of AA4.1 and B220 gave rise exclusively to B cell development and showed no evidence for self-renewal *in vivo*. The restriction of this differentiated ES cell subpopulation to the B lymphoid lineage correlates with the presence of immunoglobin rearrangements (11, 15) and Rag gene expression (11, 15, 16). Commitment to the B cell lineage upon *in vitro* differentiation is demonstrated by the rapid appearance of surface IgM$^+$ cells in the peripheral blood of reconstituted animals. The IgM$^+$ cells appeared in a single wave and no long-lived B cell reconstitution was observed. Thus, these observations suggest that stem cells or primitive multilineage progenitors are absent from the AA4.1^+ B220$^+$ ES cell population. Alternatively, the *in vitro* differentiation might induce distortions in the putative progenitor/stem cells in this subset, for example, in the expression of adhesion molecules resulting in impaired migration or survival. We think that this is unlikely, however, because multilineage potential could be readily demonstrated in our *in vitro* differentiated cell cultures.

In contrast to the exclusive and transient restoration of the B cell lineage after the transfer of AA4.1^+ B220$^+$ cells, the corresponding AA4.1^+ B220$^-$ fraction fully reconstitutes the lymphoid defect in Rag-deficient hosts. Several observations argue in favor of a primitive multilineage precursor and/or a stem cell in this population. In all experiments using several ES cell lines with different genetic backgrounds, AA4.1^+ B220$^-$ cells contributed to the pro-B cell pool, i.e., a developmental stage prior to the endogenous block of lymphocyte maturation in Rag-deficient mice. Intrathymic T cell maturation was restored for an extended period of time, suggesting a continuous replenishment of, presumably, bone marrow-derived pro-T cells. This developmental potential of *in vitro* differentiated ES cells could either be due to the transfer of an early lymphoid precursor, a primitive multilineage progenitor, or HSCs. Although the lymphoid reconstitution persisted for more than 24 weeks, a decline over time was obvious, suggesting a limitation in self-renewal capacity. This is in line with the properties of HSCs early in embryogenesis or with a progenitor activity downstream of the stem cell stage. In either case, the development of some ES-derived macrophages and c-kit$^+$ lineage-marker-negative bone marrow cells suggests multipotent precursor activity in the AA4.1^+ B220$^-$ population. Previous studies have described lymphoid reconstitution of SCID (13, 25) or Rag-deficient mice (15) with unseparated ES cells and reported the generation of mature T and B cells. In the case of Nisitani *et al.* (15), successful restoration of B cell development in the bone marrow was also observed. Although these studies provided evidence for lymphopoietic precursor activity in differentiated ES cell, the present work defines two distinct stages of lymphopoiesis during *in vitro* differentiation, both of which are able to undergo final maturation upon *in vivo* transfer.

To assess the functional competence of a lymphoid system derived entirely from ES cells, we employed a T cell-dependent immunization protocol to analyze antibody production and T cell responsiveness. Immune responses were similar, both quantitatively and qualitatively, in mice reconstituted with the homologous precursor populations isolated from ES cells or from FL. These results suggest that the ES cell-derived lymphoid system can mature to full functional competence. In addition, a direct comparison of the reconstitution potencies of both precursor populations was carried out by cotransplantation of ES- and FL-derived AA4.1^+ B220$^-$ preparations. By using allotypes and a combination of surface markers to identify the source of antibodies or cells, respectively, we consistently found a marked predominance of FL-derived cells. The decreased efficiency of ES cell progenitors might

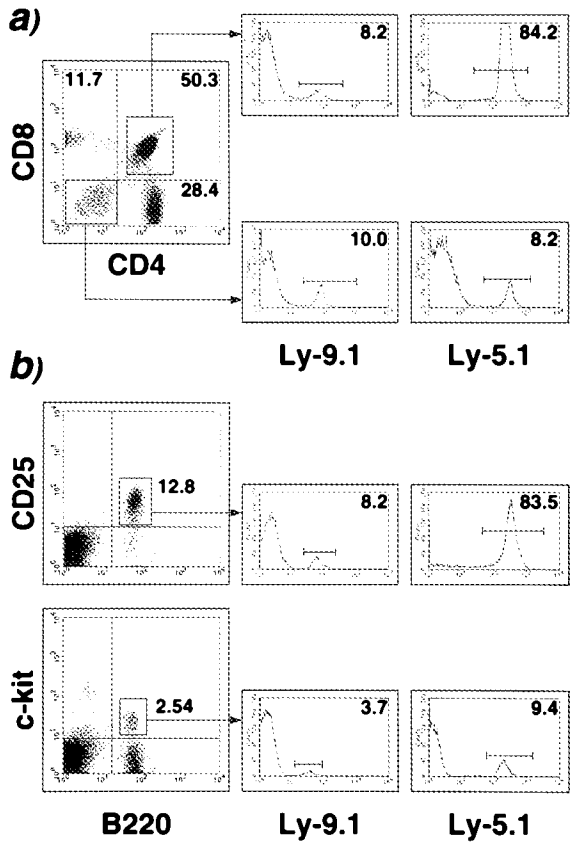

FIG. 4. Competitive reconstitution of Rag-deficient mice with ES- and FL-derived progenitors. Purified AA4.1$^+$ B220$^-$ cells from either *in vitro*-differentiated ES cells (ES cell line D3/M, Ly-9.1) or from embryonic day 14.5 FL (C57/BL6 Ly-5.1) were coinjected in sublethally irradiated Rag-1$^{-/-}$ (C57/BL6 Ly-5.2). A representative three-color cytofluormetric analysis 8 weeks after transfer of a reconstituted recipient initially injected with 4.5×10^4 ES-derived and 0.5×10^4 FL-derived cells is shown. (*a*) Thymocytes were stained with CD4/CD8 and Ly-9.1, indicative of ES-derived cells, or Ly-5.1, as a marker for FL-derived cells. B cell development was analyzed by staining surface-IgM-depleted bone marrow cells for B220/CD25 (*b Upper*) or B220/c-kit (*b Lower*). Double positive cells were analyzed by using Ly-9.1 or Ly-5.1 as a third parameter (single parameter histograms), allowing the distinction among endogenous or ES- or FL-derived lymphoid precursors. Numbers shown indicate percentages in quadrants (*a*) or individual regions (*b*).

reflect a partially deregulated gene expression pattern during *in vitro* differentiation resulting in impaired adaptation to the *in vivo* environment. Interestingly, ES cell-derived populations also contributed significantly less than expected to the CD4$^+$ CD8$^+$ cells in the thymus, which are generated by rapid proliferation. This could indicate a reduced proliferative potential of ES-derived cells *in vivo*. Our data are in line with the limited lympho-hematopoietic potential reported for unseparated ES cells (16). It is not clear from these results whether the reconstitution deficiencies of ES cells arise at the stem cell level or further downstream during *in vitro* differentiation.

To what extent do ES cells recapitulate fetal hematopoiesis *in vitro*? In birds, the definitive HSCs originate intraembryonically from the para-aortic splanchnopleural mesoderm (26) rather than from the extraembryonic yolk sac, which has been proposed to be the main source of fetal HSCs in mammals (27). The emergence of erythroid and myeloid precursors during *in vitro* differentiation of ES cells has been taken as evidence for a yolk sac equivalent of hematopoietic development (1). During embryogenesis, the yolk sac contains progenitors that can undergo *in vitro* (20, 28, 29) or upon transfer in animals (29, 30) differentiation to lymphocytes. Most recently, Cumano *et al.* (31) provided evidence for a restriction of the lymphoid potential to the intraembryonic para-aortic splanchnopleura when analyzed before the onset of circulation. An interpretation that integrates the different experimental results would, therefore, place the hematopoietic potential of differentiated ES cells at a similar developmental stage as that of the para-aortic splanchnopleura. The splanchnopleura develops at later times into the aorta–gonad–mesonephros (AGM) region. Interestingly, hematopoietic long-term repopulating activity is not present before embryonic day 10 in the AGM (25, 32), just prior to the colonization of the FL by HSCs (33). One possible explanation for the impairment of ES cells in long-term hematopoiesis could be a lack of the functional transition within the HSC pool, generating the long-term repopulating activity. *In vivo*, this transition has been mapped to the anterior AGM region (32). Experiments involving cocultures of differentiated ES cells with AGM region-derived cells might provide a suitable system to distinguish between an alteration at the stem cell level and the absence of factors provided by a specialized microenvironment. Thus, with the present study, this should be helpful in understanding the mechanism of *in vitro* hematopoiesis and might provide additional insights into the physiological process of lymphopoiesis during ontogenesis.

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